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Enhanced Production of α-Galactosyl Epitopes by Metabolically Engineered *Pichia pastoris*

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A metabolically engineered *Pichia pastoris* strain was constructed that harbored three heterologous enzymes: an S11E mutated sucrose synthase from *Vigna radiata*, a truncated UDP-glucose C4 epimerase from *Saccharomyces cerevisiae*, and a truncated bovine α-1,3-galactosyltransferase. Each gene has its own methanol-inducible alcohol oxidase 1 promoter and transcription terminator on the chromosomal DNA of *P. pastoris* strain GS115. The proteins were coexpressed intracellularly under the induction of methanol. After permeabilization, the whole *P. pastoris* cells were used to synthesize α-galactosyl (α-Gal) trisaccharide (Galα1,3Galβ1,4Glc) with in situ regeneration of UDP-galactose. Up to 28 mM α-Gal was accumulated in a 200-ml reaction. The *Pichia* system described here is simple and flexible. This work demonstrates that recombinant *P. pastoris* is an excellent alternative to *Escherichia coli* transformants in large-scale synthesis of oligosaccharides.

The methylotrophic yeast *Pichia pastoris* has been developed into a commercially important host for the production of heterologous proteins (3, 14). *Pichia* does not have the endotoxin problem associated with bacteria or the viral contamination problem of proteins produced in animal cell culture. Furthermore, *P. pastoris* can utilize methanol as a carbon source in the absence of glucose. Since the proteins expressed in *P. pastoris* are typically folded correctly, the genetically engineered *P. pastoris* provides an excellent alternative to *Escherichia coli* expression systems. A large number of proteins have been successfully expressed in this system, including mammalian glycosyltransferases, such as human β-1,4-galactosyltransferase I, α-2,6-sialyltransferase, and α-1,3-fucosyltransferase VI (15).

α-Galactosyl (α-Gal) epitopes are oligosaccharides with a terminal Galα1,3Gal sequence. They are abundantly expressed on the cell surface of mammals other than humans, apes, and Old World monkeys. In contrast, the human body naturally produces a large amount of anti-Gal antibodies (anti-Gal), specific for α-Gal epitopes. The interaction between α-Gal and anti-Gal antibodies in the serum of recipients is the main cause of hyperacute rejection in xenotransplantation (10). The potential of α-Gal epitopes for practical applications in clinical processes such as immunoadsorption (20) has led to an increased demand for an efficient approach adaptable to their large-scale synthesis.

Biotransformation by recombinant microbes has recently emerged as one of the most efficient methods in the synthesis of carbohydrates. Oligosaccharides were produced in gram scale by both *E. coli* (4–8, 13) and *Saccharomyces cerevisiae* (12) cells. In this paper, we reported the synthesis of α-Gal trisaccharide by metabolically engineered *P. pastoris* cells harboring three heterologous enzymes: the S11E mutated (16) mung bean sucrose synthase (mbSusA), the truncated UDP-glucose C4 epimerase (trGalE) from *S. cerevisiae* (11), and the truncated bovine (9) α-1,3-galactosyltransferase (α1,3GalT). MbSusA catalyzes the cleavage of sucrose to generate UDP-glucose and fructose. GalE catalyzes the conversion from UDP-glucose to UDP-galactose. Both are reversible reactions driven forward by constant consumption of UDP-galactose, in which α1,3GalT transfers the galactose residue to acceptor substrate lactose to generate α-Gal trisaccharide (Fig. 1). The focus of our study is to develop a genetically engineered *P. pastoris* system for large-scale production of α-Gal epitope with in situ UDP-galactose regeneration. We demonstrated that all genes were integrated into the *P. pastoris* chromosome and expressed as active proteins under the control of methanol-inducible alcohol oxidase 1 promoter. To our knowledge, this is the first example in which up to three heterologous enzymes are coexpressed in *P. pastoris* and the whole yeast cells are used as catalyst in the synthesis of oligosaccharides. The ability of the system to simultaneously produce multiple enzyme activities is significant because most of the commercially important biotransformations and biocoversions are multistep reactions.

MATERIALS AND METHODS

Strains, plasmids, and materials. All strains and plasmids used in this work are listed in Table 1. The restriction enzymes and Vent DNA polymerase were purchased from New England Biolabs (Beverly, Mass.). T4 DNA ligase was obtained from Promega (Madison, Wis.). The PCR purification kit, QIAEX II gel extraction kit, and DNA Miniprep spin kit were obtained from Qiagen (Santa Clarita, Calif.). UDP-D-[6-^{3}H]galactose was obtained from Sigma Chemical Co. (St. Louis, Mo.). A multicopy *Pichia* expression kit was obtained from Invitrogen Corp. (San Diego, Calif.). Other reagents were of analytical grade or higher. All kits or enzymes were used according to the manufacturers' instructions.

Construction of plasmids. The truncated *α1,3galT* gene, *mbsusA* gene, and truncated *galE* gene were first cloned into plasmid pAO815 individually. The *α1,3galT* gene was PCR amplified from plasmid pET15b-αGalT (9) by two primers: *α1,3galT*-F (5′GCGGAATTCCTTAAGATGCTATCGGACTGGTTC AACCCA) and *α1,3galT*-R (5′GCGGAATTCACTAGTTCAGACATTATTTC TAACCAC). To facilitate the cloning of two other genes, the *Afl*II and *Spe*I

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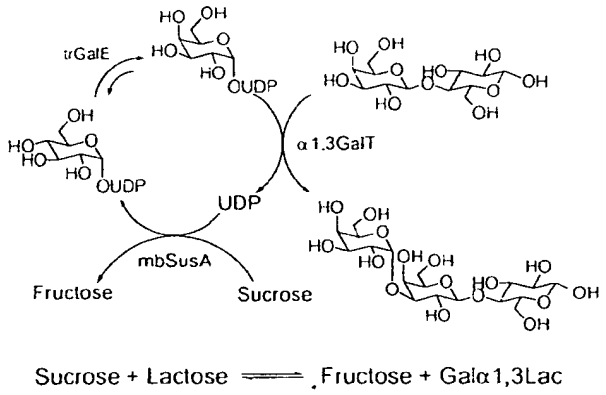

FIG. 1. The biosynthetic pathway of α-Gal trisaccharide with the regeneration of UDP-galactose.

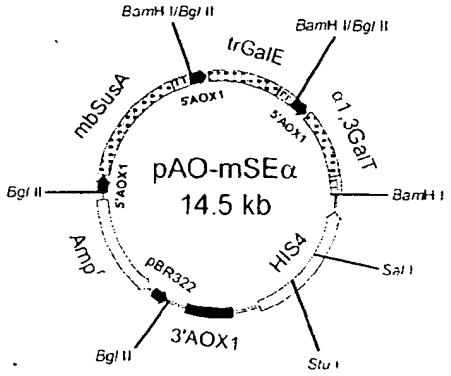

FIG. 2. Map of recombinant plasmid pAO815-mSEα.

restriction sites (underlined) were incorporated into primers *α1,3galT*-F and *α1,3galT*-R, respectively. The PCR product was inserted into the *Eco*RI restriction site of pAO815 to generate pAO815-α1,3GalT. The correct direction of the gene was verified by restriction mapping. Similarly the *mbsusA* and *trgalE* genes were PCR amplified from plasmid pED-01-S11E (16) with primers *mbsusA*-F (5'CCCCTTAAGATGGCTACCGATCGTTTGACCCGTG) and *mbsusA*-R (5' GGGACTAGTTTACTCAACAGCAAGGGGCACAGAC) and *S. cerevisiae* chromosomal DNA was amplified with primers *galE*-F (5'CCCCTTAAGATG ACAGCTCAGTTACAAAGTGAAAG) and *galE*-R (5'GGGACTAGTTCAA TCTTCAGCGGAAAATCTGGCCTC). Both PCR products were digested with *Afl*II-*Spe*I and ligated into the pAO815 fragment prepared by digestion of plasmid pAO815-α1,3GalT with the same restriction enzymes to generate plasmids pAO815-mbSusA and pAO815-trGalE. The next step was to link all three expression cassette together to form the final plasmid, pAO815-mSEα (Fig. 2). Plasmid pAO815-mbSusA was linearized by *Bam*HI and dephosphorylated. Meanwhile, plasmid pAO815-trGalE was digested by *Bgl*II and *Bam*HI. The trGalE expression cassette was gel purified and ligated with linearized pAO815-mbSusA to generate plasmid pAO815-mSE. Then, plasmid pAO815-mSE was linearized by *Bam*HI and dephosphorylated. At the same time, plasmid pAO815-trαGalT was digested by *Bgl*II and *Bam*HI. The α1,3GalT expression cassette was gel purified and ligated with linearized pAO815-mSE to generate the plasmid pAO815-mSEα. The integrity of all these plasmids was verified by restriction mapping.

Yeast strain preparation. The plasmid pAO815-mSEα was linearized by *Sal*I and transformed into *P. pastoris* strains GS115 and KM71 by the spheroplast transformation method for integration at the *HIS4* locus of the yeast genome. The transformants were spread on plates containing minimal dextrose medium (13.4 g of yeast nitrogen base with ammonium sulfate without amino acids [Invitrogen, San Diego, Calif.] liter^{-1}, 400 μg of biotin liter^{-1}, 20 g of dextrose liter^{-1}, and 15 g of agar liter^{-1}) to select His$^+$ yeast cells. The *P. pastoris* recombinants were analyzed by PCR with primer pairs (*α1,3galT*-F and *α1,3galT*-R, *mbsusA*-F and *mbsusA*-R, and *galE*-F and *galE*-R) to confirm the integration of all three genes. All experimental procedures were performed in accordance with the instruction manual (catalog no. K1750-01) from Invitrogen Corp. (San Diego, Calif.).

Composition of media. (i) BMGY and BMMY. Buffered complex glycerol medium (BMGY) contains 100 mM potassium phosphate (pH 6.0), 10 g of yeast extract liter^{-1}, 20 g of peptone liter^{-1}, 13.4 g of yeast nitrogen base with ammonium sulfate without amino acids liter^{-1}, 400 μg of biotin liter^{-1}, and 10 ml of glycerol liter^{-1}. For buffered complex methanol medium (BMMY), the glycerol was substituted for by 5 ml of methanol liter^{-1}.

(ii) PTM1 trace salts. PTM1 trace salts medium contained 6 g of cupric sulfate·5H_2O liter^{-1}, 0.08 g of sodium iodide liter^{-1}, 3 g of manganese sulfate·H_2O liter^{-1}, 0.2 g of sodium molybdate·2H_2O liter^{-1}, 0.02 g of boric acid liter^{-1}, 0.5 g of cobalt chloride liter^{-1}, 20 g of zinc chloride liter^{-1}, 65 g of ferrous sulfate·7H_2O liter^{-1}, 0.2 g of biotin liter^{-1}, and 5 ml of sulfuric acid liter^{-1}.

(iii) Basal salt medium. Basal salt medium contains 26.7 ml of phosphoric acid liter^{-1}, 1 g of calcium chloride·2H_2O liter^{-1}, 18.2 g of potassium sulfate liter^{-1}, 14.9 g of magnesium sulfate·7H_2O liter^{-1}, 4.13 g of potassium hydroxide liter^{-1}, 4.4 ml of PTM1 trace salts liter^{-1}, and 40 g of glycerol liter^{-1}.

Culture conditions. To prepare the inocula, yeast cells were transferred from YPD agar plates into 250-ml Erlenmeyer flasks containing 50 ml of BMGY and grown at 28°C in C25 incubator shaker (New Brunswick Scientific, Edison, N.J.) at 300 rpm. The cells were harvested in log phase and then used in flask or fermentor cultivations.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference[b]
Strains		
E. coli DH5α	*lacZΔM15 hsdR recA*	Gibco
S. cerevisiae 2601		ATCC
P. pastoris GS115	*His4* Mut$^+$	Invitrogen
Plasmids		
pET15b-αGalT	Amp^r, pET15b derivatives containing truncated bovine *α1, 3galT* gene	9
pED-01-S11E	Amp^r, pET21d derivatives containing a mutated *mbsusA* gene	16
pAO815	Amp^r, *Pichia* expression vector	Invitrogen
PAO815-α1,3GalT	Amp^r, pAO815 derivatives containing truncated bovine *α1, 3galT* gene	This work
PAO815-trGalE	Amp^r, pAO815 derivatives containing truncated *S. cerevisiae galE* gene	This work
PAO815-mbSusA	Amp^r, pAO815 derivatives containing a mutated *mbsusA* gene	This work
PAO815-mSE	Amp^r, pAO815 derivatives containing both *mbsusA* and *galE* genes	This work
pA0815-mSEα	Amp^r, pAO815 derivatives containing *mbsusA*, *galE* and *α1, 3galT* genes	This work

[a] Mut$^+$, methanol utilization positive; Amp^r, ampicillin resistant.
[b] Gibco, Gibco-BRL Life Technology, Carlsbad, Calif; ATCC, American Type Culture Collection, Manassas, Va.; Invitrogen, Invitrogen Corp., San Diego, Calif.

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(i) Flask cultivations. The flask cultivations were performed in 500-ml Erlenmeyer flasks at 28°C in C25 incubator shaker (300 rpm). The cell pellet was resuspended to an optical density at 600 nm (OD_{600}) of 1.0 in 50 ml of BMMY to induce expression. Filter-sterilized 100% methanol was added to a final concentration of 0.5 to 1.5 ml liter^{-1} daily to maintain induction.

(ii) Fermentor cultivations. The expression of recombinant enzymes was performed at 28°C in BIOSTAT B fermentor (B. Braun Biotech, Int., Melsungen, Germany) with a 2-liter water-jacketed glass vessel. All fermentations began in 1 liter of basal salt medium with glycerol as a carbon source to obtain a high cell density. After depletion of the glycerol, filter-sterilized methanol containing 10 ml of PTM1 trace salts liter^{-1} was added daily to a final concentration of 0.75 ml liter^{-1} to maintain induction. After 96 h of induction, cells were harvested by centrifugation (4,000 × *g*, 20 min, 4°C), washed twice with Tris-HCl (50 mM [pH 7.0]), and frozen and thawed three times before use in the reaction.

Biomass and protein analysis. The OD of the broth was measured against BMMY at 600 nm by a UV spectrophotometer (HP 8453; Hewlett-Packard). The samples were diluted appropriately to ensure a value within the linear range. The cells were collected by centrifugation and resuspended in chilled lysis buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 0.5% [wt/vol] Triton X-100, 10% [wt/vol] glycerol). After disruption by brief sonication (Branson Sonifier 450; VWR Scientific) on ice, the lysate was cleared by centrifugation (12,000 × *g*, 20 min, 4°C). The concentration of protein was determined according to the method of Bradford (2).

Enzyme activity assays. In this study, 1 U of enzyme activity was defined as the amount of enzyme that produced 1 μmol of product per min at 30°C.

(i) Sucrose synthase. The sucrose synthase activity was measured in the cleavage direction (formation of UDP-Glc and fructose from sucrose and UDP). Each reaction mixture contained the following components in a final volume of 100 μl: 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM dithiothreitol, 100 mM sucrose, 10 mM UDP, and the sample to be assayed. After 30 min of incubation at 30°C, the reactions were quenched by placing the mixtures in boiling water for 2 min. Samples were analyzed by capillary electrophoresis (ISCO model 3850 capillary electropherograph).

(ii) UDP-glucose C4 epimerase. For UDP-glucose C4 epimerase reactions, the 50-μl (total volume) reaction mixtures consisted of 1 mM UDP-Glc, 20 mM Tris-HCl (pH 8.0), and various amounts of enzyme. The mixtures were incubated at 30°C for 15 min, and the reactions were quenched by placing the samples in boiling water for 5 min. Samples were analyzed by capillary electrophoresis (ISCO model 3850 capillary electropherograph).

(iii) Galactosyltransferase. The activity of α-1,3-galactosyltransferase was assayed according to a previously published protocol (9).

Synthesis of α-Gal trisaccharide with yeast cells. The yeast cells were permeablized by three freeze-thaw cycles before use in the reaction. The reaction mixture (200 ml) contained yeast cells (50 g), sucrose (13.6 g, 40 mmol), lactose (5.3 g, 15 mmol), UDP (0.16 g, 0.4 mmol), $MgCl_2$ (0.38 g, 4 mmol), and dithiothreitol (60 mg, 0.4 mmol) in MES (morpholineethanesulfonic acid) buffer (100 mM [pH 6.5]). The reaction mixture was incubated at 30°C for 48 h. The progress of the reaction was monitored by thin-layer chromatography and high-performance liquid chromatography as previously described (5). After the reaction, the cells were removed by centrifugation (6,000 × *g*, 20 min). The remaining sucrose and lactose were hydrolyzed to monosaccharides by incubating the supernatant with invertase (20 mg; Sigma) and β-galactosidase (80 mg; Sigma) for 8 h at 25°C. The mixture was then poured onto a column packed with graphitized carbon (Supelco, Inc., Bellefonte, Pa.). The column was washed with water, and the trisaccharide product was eluted with H_2O-ethanol (1:1 [vol/vol]). The product was characterized by nuclear magnetic resonance (NMR) and mass spectrometry. Selected ^1H NMR (500 MHz, D_2O): δ 3.99 (m, 2H), 4.35 (d, J = 7.6 Hz, 1H), 4.48 (d, J = 7.8 Hz), 4.96 (d, J = 3.7 Hz, 1H), 5.04 (d, J = 3.5 Hz). ^{13}C NMR (125 MHz, D_2O): δ 102.83, 95.76, 95.38, 91.77, 78.67, 78.46, 77.15, 75.04, 74.73, 74.34, 73.69, 71.40, 71.08, 70.80, 70.04, 69.49, 69.21, 69.08, 68.17, 64.76, 61.01, 60.89, 60.08, 59.97. High-resolution fast atom bombardment mass spectrometry calculated for $C_{18}H_{32}O_{16}$ (M + Na): 527.1588. Found: 527.1582.

RESULTS

Construction of recombinant *P. pastoris* strain. The expression plasmid pAO815-mSEα (Fig. 2) was successfully constructed for the coexpression of three enzymes. Each gene has its own methanol-inducible alcohol oxidase 1 promoter (AOX1) and transcription terminator. The plasmid also contains an ampicillin resistance gene (Amp^r) and *HIS4* fragment for targeted chromosomal integration of the whole expression cassette. Restriction mapping verified that only one copy of each gene was present on the vector. After linearization at *HIS4* site by *Sal*I, the pAO815-mSEα fragment was transformed into *P. pastoris* strain GS115. Transformants were collected from selective spheroplast regeneration dextrose plates. PCR examination (Fig. 3a) showed that all three genes have been integrated into the chromosome of *P. pastoris* strain GS115.

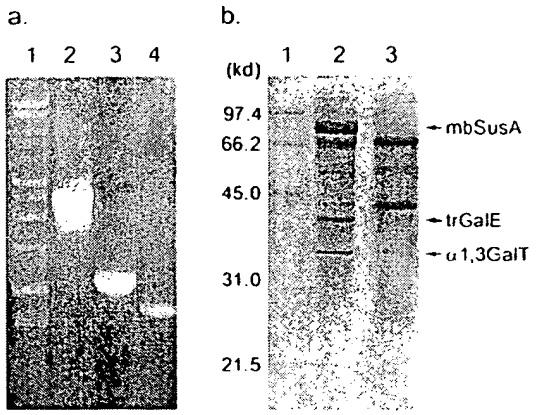

FIG. 3. (a) PCR analysis of *Pichia* integrants. Lanes: 1, 1-kb DNA ladder; 2, mbSusA; 3, trGalE; 4, α1,3GalT. (b) SDS-PAGE (12% polyacrylamide) indicating the coexpression of the enzymes in *P. pastoris*. Lanes: 1, low-range molecular weight markers (Bio-Rad); 2, mbSusA, trGalE, and α1,3GalT in pAO815-mSEα integrants; 3, *P. pastoris* GS115.

Coexpression of recombinant proteins in *P. pastoris*. Heterologous expression of recombinant enzymes was initially assessed in flask cultivations with BMMY. Methanol was added daily at a concentration between 0.5 and 1.5% (vol/vol) to maintain induction. The expressed proteins were entrapped within the *Pichia* cells, because none of them was linked to a secretion signal sequence. The yeast cells were harvested after 72 h for protein analysis and the activity assay of the key enzyme, sucrose synthase. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Fig. 3B) revealed that all three recombinant proteins were well expressed in *P. pastoris* GS115 cells. As shown in Fig. 4, variation of methanol concentration caused less difference in cell growth (OD_{600} was between 11.3 and 12.0) than in soluble protein concentration (between 57 and 76 mg liter^{-1}) and sucrose synthase activity (between 3.8 and 6.2 U liter^{-1}). The highest mbSusA activity (6.2 U liter^{-1}) was achieved with a 0.75% addition. The 2-liter fermentation was run with a selected transformant, SEα-G4, that demonstrated a high level of protein expression and enzyme activities. Samples were withdrawn every 12 h for OD measurement, determination of soluble protein, and enzyme activity assays. The yeast cells grew fast in basal salt medium (Fig. 5a). The glycerol was completely consumed within 24 h when an OD_{600} of around 20 was reached. The induction phase was then initiated by adding methanol to a final concentration of 0.75 ml liter^{-1}. The intracellular soluble protein concentration (Fig. 5a) and the enzyme activities (Fig. 5b) increased quickly during the methanol induction. The highest protein concentration (265 mg liter^{-1}) and mbSusA

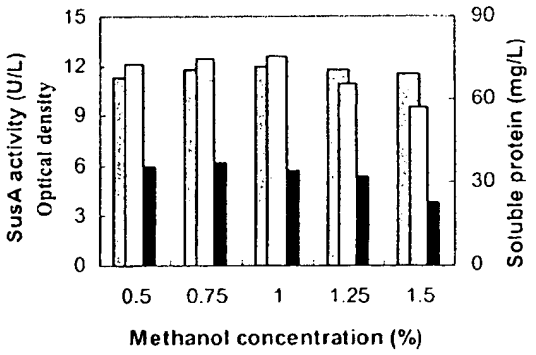

FIG. 4. Effect of methanol concentration on expression in flask cultivations with BMMY medium. Gray bars, OD_{600}; open bars, soluble protein; solid bars, sucrose synthase activity.

activity (17.9 U liter^{-1}) were obtained at about 80 h. Both of the values were three times higher than those from flask cultivations. The mbSusA and α1,3galT activities decreased slightly at the end of the fermentation.

Synthesis of α-Gal trisaccharide by *P. pastoris* cells. Small-scale (1 ml) reactions were run to analyze the effects of the concentration of donor substrate sucrose and acceptor substrate lactose on the synthesis of α-Gal (Table 2). Unlike the sucrose synthase from *Anabaena* sp., which has a K_m of 303 mM for sucrose (17), the S11E mutated sucrose synthase (mbSusA) from *Vigna radiata* Wilczek has a much higher apparent affinity (K_m = 23 mM) to sucrose (16). Therefore, a relatively lower concentration (200 mM) of sucrose was

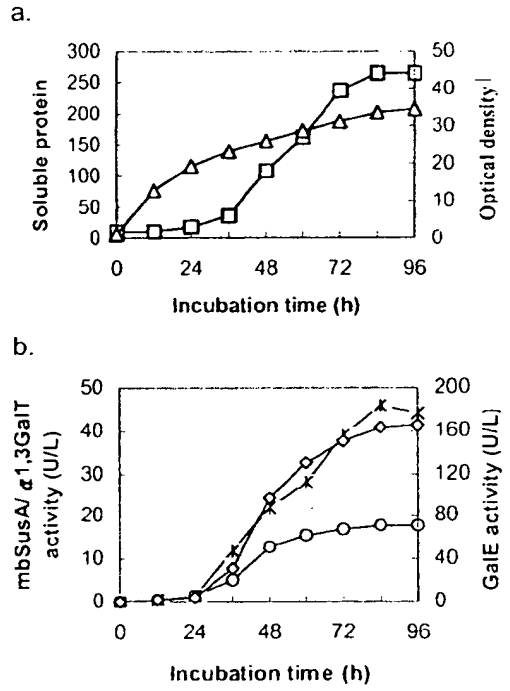

FIG. 5. Expression of recombinant enzymes in basal salt medium. △, OD_{600}; □, soluble protein; ○, sucrose synthase; ◇, UDP-glucose C4 epimerase; *, α-1,3-galactosyltransferase.

TABLE 2. Effect of substrate concentration on α-Gal production

Expt	Substrate concn (mM)		α-Gal production (mM)
	Sucrose	Lactose	
1	500	200	26.9
2	400	200	26.4
3	300	200	27.9
4	200	200	27.1
5	100	200	14.5
6	200	200	27.0
7	200	150	27.2
8	200	100	26.9
9	200	75	26.6
10	200	50	17.4

enough to push the reversible reaction toward the cleavage direction. The total yield of α-Gal trisaccharide decreased significantly when less than 100 mM sucrose was present in the reaction. Meanwhile, the *P. pastoris* whole-cell system also needed a lower concentration (75 mM) of acceptor lactose than the previously described *E. coli* cell system (200 mM of lactose), although only 17.4 mM final product had been achieved when less than 50 mM of lactose was added to the reaction (Table 2). The scale-up reaction (200 ml) was run at 30°C in a 500-ml capped Erlenmeyer flask. Time course studies (Fig. 6) indicated that the reaction reached a plateau (28 mM α-Gal) after 40 h, corresponding to 2.8 g of trisaccharide product. A total of 2.58 g of α-Gal trisaccharide product was finally recovered from the reaction mixture.

DISCUSSION

Previously we reported the synthesis of α-Gal trisaccharide by an *E. coli* strain (5), which was transformed with a single plasmid containing the sucrose synthase from *Anabaena* sp., UDP-glucose C4 epimerase from *E. coli* K12, and bovine α-1,3-galactosyltransferase. Relatively high yields were achieved with low cost. However, the recombinant *E. coli* system still suffered from three major drawbacks. First, the choice of sucrose synthase from *Anabaena* sp., a filamentous heterocystous cyanobacterium, was solely based on the compatibility consideration that *E. coli* is a prokaryotic expression system. This enzyme has a K_m of 303 mM for sucrose; therefore, a high concentration of sucrose (500 mM) was used to force the reversible reaction toward the UDP-glucose-forming direction. After the reaction, most of the sucrose remained in the mix-

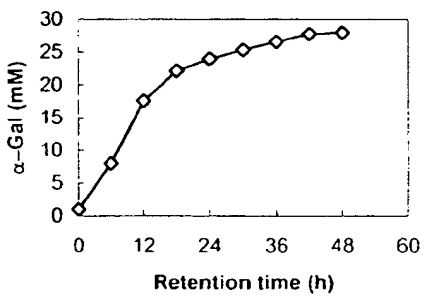

FIG. 6. Time course of the synthesis of α-Gal trisaccharide.

ture, thereby complicating the subsequent purification steps. The second apparent drawback is the addition of ampicillin during cell culture and enzyme expression to keep the selection pressure. This may hinder or at least narrow its possibility of commercialization. Finally, plasmid pLDR20 is a temperature-sensitive vector that enabled high temperature (40°C) induction and eliminated the need for chemical inducers (5). High temperature, however, may affect the solubility and stability of recombinant proteins heterologously expressed in *E. coli*. It is well established that the high-level expression of recombinant proteins at high temperature can result in the formation of insoluble aggregates known as inclusion bodies (18, 19), and a lower expression temperature may sometimes increase the activity of proteins (1).

The genetically engineered *P. pastoris* described here tend to solve these problems. The S11E mutant sucrose synthase (16) from mung bean (*V. radiata*) has a relatively low K_m (23 mM) for sucrose and high catalytic efficiency (k_{cat}/K_m, 16.5×10^{-3} s^{-1} mM^{-1}). The expression level was high in the eukaryotic *Pichia* system, and most of the proteins are soluble. As a result, the concentration of sucrose used in the reactions has dropped from 500 mM to 200 mM. Another advantage is that all three genes were integrated into the chromosomal DNA of host *Pichia* cells. The integrants were selected and maintained by their ability to grow on histidine-deficient medium with methanol as both the sole carbon source and the inducer. Therefore, antibiotics were eliminated during the fermentation processes. Moreover, the lower growth temperature (28°C) may decrease the speed of protein biosynthesis and consequently help the correct folding of recombinant enzymes within *Pichia* cells.

In the α-Gal trisaccharide synthetic reactions, sucrose and lactose serve as the donor and the acceptor substrates, respectively. Sucrose can be easily degraded by invertase, while lactose can be hydrolyzed by β-galactosidase. Both enzymes exist in many species of yeast cells, including the widely used expression host *S. cerevisiae*. Fortunately, neither of the enzymes is present in *P. pastoris*, making it an ideal metabolism-engineering host for the biocatalytic synthesis of oligosaccharides. The recombinant *Pichia* system described here is simple and flexible. By replacing the glycosyltransferase gene, novel *P. pastoris* transformants can be constructed to synthesize other galactosides. Work is in progress to incorporate a truncated bovine β-1,4-galactosyltransferase into the expression cassette. The selected *P. pastoris* integrants will be used to synthesize LacNAc disaccharide (Galβ1,4GlcNAc) by using *N*-acetylglucosamine (GlcNAc) as the starting material.

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